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Cy 1

Experimental immunization with live strains of Bacterium tularense of low virulence. First report.

by E. Gotschlich, Said Bilal Golem and Tahsin Berkin.

Turk Hifzissihha Ve Tecrubi Biyoloji Mecmuasi, 2: 145-156 (1940-41).

Among the strains of Bact. tularense made available to us by other institutes in 1936 on the occasion of the first report of tularemia in Turkey (cf. E. Gotschlich and Tahsin Berkin, Türkische Zeitschr. f. Hygiene u. exper. Biol. 1:1:129:1938), there were two that were designated as apathogenic or weakly pathogenic for laboratory animals, namely:

"Berlin 38" from Prof. Dr. Gildemeister, Vice President of the R. Koch Institute at Berlin, and "Stockholm 4" from Prof. Dr. Olin, Stockholm.

Both cultures had originally been grown in the USA and, according to available data, had been apathogenic or weakly pathogenic for laboratory animals from the start, without having been reduced to this low virulence intentionally by means of external treatment. Whether or not we were dealing here with two different transplants of the same strain, a theory apparently supported by the uniformity of their effect on laboratory animals in tests to be described later, could not be clarified by inquiries with the senders. The re-examination of both cultures for morphological, cultural and serologic characteristics conducted by us previously revealed identical action (cf. E. Gotschlich and Tahsin Berkin, op. cit. p. 130).

The subject of the investigations to be described below was twofold.

1) Examination of ~~their~~ pathogenic or apathogenic behaviour in connection with the customary laboratory animals (guinea pigs, rabbits and white mice) by various modes of infection.

2) Examination of their immunizing effect in the indicated animals upon application of live cultures. The latter tests are of particular interest, especially for the practical feasibility of vaccination, since the superiority of live vaccines over killed vaccines has been established unequivocally in the case of other infectious pathogens (e.g. plague bacilli by Strong). The test for possibly activated immunity was made with the virulent strain "Gülhane," obtained by Prof. Dr. Kemal Hüseyin in 1936 at Istanbul from a patient from Thrace.

Well-developed surface cultures of 48 hours on Francis' medium, containing agar, cystine, dextrose and horse blood, were used in connection with the apathogenic and pathogenic strains of Bact. tularense.

Tests.

Immunization of guinea pigs of about 500 g body weight by intraperitoneal inoculation of 1 loop each of a live, weakly pathogenic culture of Bacterium

tularense, strain "Stockholm 4" in intervals of about 3 weeks.

In the period 9-12 January, on 28 January and on 19 February 1939, a total of 31 guinea pigs were treated in this manner. Of these, 4 died intercurrently (*) of common pneumonia and pleuropneumonia caused by pneumococci and streptococci, while bacteriological examinations for Bact. tularense proved negative. An additional 12 animals succumbed to peritonitis, 10 of these within 12-24 hours after the first, two others within 12-24 hours after the third injection; in 5 of these 12 animals Bact. tularense was demonstrated culturally. An endotoxic effect of Bact. tularense apparently is involved in the case of these 12 animals which died immediately after the intraperitoneal injection, whereas the viable tularemia bacilli demonstrated in 5 cases had escaped destruction owing to the attenuation of organic defenses. The fact that the lethal effect is exerted either immediately after injection or not at all, leads to the conclusion that a genuine infection with the multiplication of the pathogen in the organism is not involved.

(*) The frequency of these intercurrent cases in our test series approximated that of our entire animal stock.

After the deduction of 4 animals that died intercurrently and 12 that succumbed to the effects of endotoxin, 15 remained as survivors of the threefold intraperitoneal immunization with 1.0 loop of culture. Of these 15 animals, 4 were sacrificed 3 weeks after the last treatment and examined for signs of a possible chronic infection, at which time 1 nodule was found in the liver in 3 cases, 2 of them with swelling of the lymph glands, and a small abscess in the epiploon in one case. Bacteriological examinations of these lesions for Bact. tularense were invariably negative. The remaining 14 animals were tested for the state of immunity 15 days after the last treatment, by infection with calibrated amounts of a virulent culture of Bact. tularense, with the following results:

Table I.

Intraperitoneal inoculation of:		Control animals (without prior immunization)	
1.0 loop:	dies of tularemia after 2 days	1.0 loop:	dies of tularemia after 2 days
0.1 "	survives	0.1 "	" 3 days
0.1 "	survives	0.1 "	" 3 days
0.01 "	survives	0.01 "	" 3 days
0.001 "	survives	0.001 "	" 4 days
Conjunctival inoculation of:			
1.0 loop:	dies intercurrently of pneumonia (bacteriol. exam. f. tular. neg.)	1.0 loop:	dies of tularemia after 14 days
1.0 "	survives	0.1 "	" 13 days
0.1 "	survives		Results of section and bacter. exam. f. tularemia positive in both animals
0.1 "	survives		

Cutaneous inoculation by rubbing into the shaved abdominal skin:

1.0 loop: survives	1.0 loop: dies of tularemia after 8 days
1.0 " survives	0.1 " survives
	0.01 " survives
	0.001 " survives.

Immunization tests with guinea pigs by intraperitoneal inoculation with 1.0 loop each of a live, weakly pathogenic culture of Bact. tularense "Berlin 38" in intervals of about 3 weeks, on 21 Jan., 15 Feb. and 2 Mar. 1939.

Of 11 pre-treated animals, 3 die within 12-24 hours after the first injection, accompanied by peritonitis and positive bacteriological findings of Bact. tularense, apparently by the effect of endotoxin.

Of the 8 surviving animals, 2 are sacrificed 14 days after the last treatment and examined for a possible chronic infection with tularemia: One of these animals shows completely negative post mortem and bacteriological findings. Testing of the remaining 6 animals with the virulent culture "Gülhane" on 16 March 1939, i.e. 14 days after the last treatment, has the following results:

Table II.

Intraperitoneal inoculation of:	Control animals (without prior immunization)
0.1 loop: survives	0.1 loop: dies of tularemia after 2 days
0.01 " survives	0.01 " " 3 days
	In both animals, positive post mortem and cultural findings of tularemia.
Conjunctival inoculation of:	
1.0 loop: survives	1.0 loop: dies of tularemia after 7 days
0.1 " survives	0.1 " " 10 days
	Autopsy positive in both animals. In the first, cultural findings of Bact. tularense.
Cutaneous inoculation of:	
1.0 loop: survives	1.0 loop: dies after 15 days
0.1 " survives	0.1 " " 8 days.
	In both animals, positive autopsy for tularemia; cultural demonstration of Bact. tularense fails.

In the preceding, we have first reported the results of these two test series with the strains "Stockholm 4" and "Berlin 38" separately, in order to show the uniformity of the results, which also extends to the number of

animals succumbing to the endotoxic effect (in the case of "Stockholm 4", among 27 animals, after the deduction of intercurrent deaths: 12, i.e. 44%, in the case of "Berlin 38," among 11 pre-treated animals: 3, i.e. 27% died of endotoxin).

The summation of these two tests shows the following very distinct immunizing effect on animals surviving a threefold intraperitoneal injection of 1.0 loop each of a weakly pathogenic culture, i.e. a very rigorous treatment, against the indicated infection with virulent culture by different modes of instillation.

Table III.

Immunized animals.	Control animals without prior immuniz.
Intraperitoneal infection (7 animals)	**Intraperitoneal infection (7 animals)**
1.0 loop: dies of tularemia after 2 days	1.0 loop: dies of tularemia after 2 days.
0.1 "	0.1 "
0.1 " all 3 survive	0.1 " all 3 die of tularemia
0.1 "	0.1 " after 3 days
0.01 "	0.01 " both die of tularemia
0.01 " all 3 survive	0.01 " after 3 days
0.001 "	0.001" dies of tularemia after 4 days
Conjunctival infection (6 animals)	**Conjunctival infection (4 animals)**
1.0 loop: both die of tularemia	1.0 loop: dies of tularemia after 7 days
1.0 "	1.0 loop: dies of t. after 14 days
1.0 " this animal dies intercurrently from pneumonia after 9 days; autopsy & bacteriol. exam. for tularemia negative.	0.1 " " 10 days
	0.1 " " 13 days
0.1 loop:	
0.1 " all 3 survive.	
0.1 "	
Cutaneous infection (4 animals)	**Cutaneous infection (6 animals)**
1.0 loop:	1.0 loop: dies of tularemia after 7 days
1.0 "	1.0 " " 10 days
0.1 " all survive	0.1 " " 10 days
0.1 "	0.1 "
	0.1 " all survive
	0.01 "
	0.001 "

The summary reveals that the obtained active immunity conveys a positive protection against the large dose of 1.0 loop in cutaneous and conjunctival infection, and against intraperitoneal infection up to a dose of 0.1 loop, while all non-treated control animals succumb to doses of 0.001 intraperitoneally and 0.1 per conjunctiva, and that the cutaneous infection with 0.1 loop apparently represents the limit of lethal dosage and that the smaller doses of 0.01 and 0.001 loop are tolerated.

During the discussion of tests with strains "Stockholm 4" and "Berlin 38," we have already treated the possibility of dealing with chronically or latently infected animals; post mortem findings were completely negative in 2 out of 6 animals examined 3 weeks after the last injection; furthermore: The splenic tumescence characteristic of tularemia was absent in all immunized animals and cultural examinations for Bact. tularense were negative. A confirmation by negative results of organ infusion from one guinea pig to another, attempted once with a negative outcome, was futile in view of the weakly pathogenic condition of our test strains. Particular interest is offered by the post mortem findings of 5 guinea pigs who had been immunized in preliminary tests in July 1938, had survived, and had been dissected and examined bacteriologically in the beginning of March 1939, i.e. about 8 months later. Here, too, the cultural result was invariably negative; the autopsy showed a completely negative result in one case, while the following small lesions were found in the other 4 animals:

1) 1 necrotic spot in the liver and adhesions between liver and plexus.
2) 1 nodule in the lung.
3) 1 small hepatic abscess with caseous pus.
4) 1 large nodule in the liver.

In the latter two cases, as well as in some of the post mortem findings from our main tests with cultures "Berlin 38" and "Stockholm 4" described above, we still hesitate to rule out the possibility of a chronic infection, notwithstanding the negative bacteriological results.

Concerning the dangerousness of the three modes of infection tested by us, the intraperitoneal mode occupies the first place, followed by the conjunctival infection (as evidenced by the longer course); the cutaneous mode is last. The latter contrasts with the plague bacillus, where the cutaneous infection frequently is superior to the intraperitoneal. The relationship of Bact. tularense with B. pestis therefore consists only in their epidemiological factor and in post mortem findings, but not extending to morphologic-cultural and serologic facets, as already pointed out by E. Gotschlich and Tahsin Berkin (op. cit. p. 133).

The active immunity obtained by treatment with live, weakly virulent cultures of Bact. tularense is superior by far to immunisation with killed, virulent culture, as shown by the following tests.

7 guinea pigs are inoculated intraperitoneally 3 times with 14-day intervals (on 10 March, 25 March and 8 April 1939) with 1.0 loop each of a virulent culture of Bact. tularense (strain Gülhane) killed by the effect of

60°C for one hour. The treatment is well tolerated (without losses from endotoxic effects); of course the immunizing effect is also much lower, as determined by tests with a live, virulent culture of "Gülhane" on 28 April 1939, i.e. 3 weeks after the last pre-treatment:

Table IV.

Intraperitoneal injection (3 animals)	Control animals not pre-treated. Intraperitoneal injection (3 animals)
0.1 loop: dies of tularemia after 6 days	0.1 loop: dies of tularemia after 3 days
0.01 " " 8 days	0.01 " " 5 days
0.001 " " 12 days	0.001 " " 6 days.
Conjunctival infection (2 animals)	Conjunctival infection (2 animals)
0.1 loop: survives	0.1 loop: dies of tularemia after 8 days
0.01 " dies of tularemia after 14 days	0.01 " survives after chronic infection. Conjunctivitis & glandular swelling.
Cutaneous infection (2 animals)	Cutaneous infection (2 animals)
1.0 loop: survives	1.0 loop: survives
0.1 " survives	0.1 " survives

Post mortem and cultural examinations of guinea pigs killed by tularemia were always positive.

Tests with rabbits immunized with 3 intraperitoneal injections of 1.0 loop each of "Stockholm 4" on 9 January, 28 January and 15 February 1939.

Of 6 animals treated in the above manner, 1 dies after 2 days with positive findings of Bact. tularense (endotoxic effect). Of the 5 survivors, 3 are sacrificed on 23 March 1939 for the purpose of obtaining serum. The serum shows an agglutination titer of between 80 and 320 against Bacterium tularense and 0-40 against Brucella Bang (sympathetic agglutination), and lacks immunity against simultaneous infection with a virulent culture of tularemia in the animal test. 8 rabbits were inoculated subcutaneously with 5.0 ccm of this serum and were simultaneously tested with the following doses of a virulent culture:

Table V.

4 rabbits, cutaneous instillation of	1.0 loop	
	1.0 "	all succumbed to tularemia
	0.1 "	after 8-13 days
	0.1 "	

4 rabbits, conjunctival infection with 1.0 loop
1.0 " all succumbed to tularemia
0.1 " after 7-14 days.
0.1 "

Autopsy invariably positive.
Cultural demonstration positive in 3 cases.

The two remaining rabbits are tested on 29 April 1939 for active immunity against intraperitoneal injection of 1.0 loop of a virulent culture. They survive.

Tests with white mice immunized with 3 injections of 0.5 loop each of "Stockholm 4" on 9 January, 28 January and 15 February 1939.

Of 15 mice treated in the indicated manner, 2 die after 5 days (one of them with positive cultural findings of Bact. tularense), 2 others after 10 days and 1 after 13 days (the latter 3 without bacteriological confirmation).

Of the 10 surviving mice, 2 die following the second injection, after 14 and 15 days, respectively; the latter due to a mechanical accident, but with positive cultural findings of Bact. tularense.

The surviving 8 mice tolerate the third injection without complications and are tested for active immunity against the following doses of virulent culture 14 days later:

Table VI.

Immunized animals.	Controls.
Intraperitoneal injection (2animals)	Intraperitoneal injection (2 animals)
0.01 loop: dies after 4 days with positive Bact. tularense	0.01 loop: dies after 4 days with positive Bact. tularense
0.001 " survives	0.001 " dies after 4 days without cultural findings.
Subcutaneous injection (2 animals)	Subcutaneous injection (2 animals)
0.01 loop: survives	0.01 loop: dies after 4 days without cultural findings.
0.001 " survives	0.001 " dies after 4 days with positive cultural findings.
Conjunctival infection (2 animals)	Conjunctival infection (2 animals)
0.01 loop: survives	0.01 loop: dies after 2 days without cultural findings.
0.001 " survives	0.001 " dies after 1 day without cultural findings of Bact. tularense.

Cutaneous infection (2 animals)	Cutaneous infection (2 animals)
0.01 loop: survives	0.01 loop: dies after 5 days without cultural findings.
0.001 " survives	0.001 " dies after 6 days with positive findings of Bact. tularense.

The result of these tests is quite clear: While all non-treated control mice succumbed to the infective dose of 0.001 loop of virulent culture via the intraperitoneal, conjunctival, subcutaneous and cutaneous application, the animals actively immunized with live, weakly pathogenic culture survive the subsequent infection with the tenfold dose of a virulent culture, with the single exception of one mouse inoculated with 0.01 intraperitoneally. However, this immune effect is achieved at the expense of almost one-half the animal contingent. We are not dealing here with an endotoxic effect, as had been the case with our guinea pigs, and which took place within the first 24 hours after injection, but with a genuine infection, as revealed by the protracted course of 5-15 days. While the dose of 0.5 loop utilized in subcutaneous pre-treatment was enormous for the mouse, it was important to show initially, as in the tests with guinea pigs, that a positive immunity may be achieved in the surviving animals by the utilization of very large doses — regardless of losses. A more sparing method must naturally be developed for practical application, with smaller doses and with instillation at a less vulnerable portal of entry. Our second report will treat the preceding questions.

Summary of results.

1) The two strains of Bact. tularense "Berlin 38" and "Stockholm 4," weakly pathogenic for laboratory animals, are able to evoke a genuine infection in about one-half of white mice — the experimental animal most susceptible to tularemia — when inoculated subcutaneously with ½ loop of a bacterial suspension.

2) In larger laboratory animals, a massive intraperitoneal injection of 1.0 loop causes a fatal endotoxic effect only within the first 24 hours after application, in about 16% of rabbits and an average of 40% of guinea pigs tested with the two strains of Bact. tularense indicated.

3) The comparison of infective modes with virulent culture by intraperitoneal, conjunctival and cutaneous means shows that the intraperitoneal application is the most effective, the cutaneous mode the last dangerous, and the conjunctival infection occupies the median.

4) Concerning the relatively low effectiveness of the cutaneous infection by rubbing into the shaved abdominal skin, as compared with that of the intraperitoneal infection, Bact. tularense is contrasted with the plague bacillus to which it is related only in respect to epidemiology and common sectional characteristics, while it resembles the brucella group more closely with regard to morphologic, cultural and serologic considerations.

5) The conjunctival infection of test animals most closely duplicates the natural conditions of human infection, in which the oculo-glandular type of disease is involved in over 1/3 of all cases, according to the world literature and our own experiences in Thrace.

This natural disease picture may also be reproduced in the test animal by infection emanating from the conjunctiva, resulting first in conjunctivitis (which may even heal in immunized animals), then in the swelling of regional lymph glands and finally, in general infection.

6) The animals surviving our thoroughgoing method of immunization (1.0 loop suspension for rabbits and guinea pigs, intraperitoneally, — 0.5 loop for white mice, subcutaneously) reveal an extensive, active immunity which fails only in the case of large doses given intraperitoneally.

7) This immunity, obtained by prior treatment with live, weakly pathogenic strains of Bact. tularense is far superior to the incomplete protection achieved by the application of killed culture. Endotoxin can no longer be demonstrated in these inactivated cultures.

8) The possibility that chronic or latent infections (so-called "premunition") might be involved in the case of our animals inoculated with live, weakly virulent material, is to be negated for a number of our cases, judging from the negative results of autopsies and bacteriological examinations of our immunized animals, but must be conceded in certain cases.